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LOW TEMPERATURE EVOKES RAPID EXOCYTOSIS OF SYMBIOTIC ALGAE BY A SEA ANEMONE

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ABSTRACT

Darkness evokes expulsion of zooxanthellae from the sea anemone *Aiptasia pulchella,* but brief exposure to low temperature (4°C, 4 h) increases the expulsion rate four-fold. Ninety-eight percent of the zooxanthellae are expelled within four days. Low temperature incubation had no detectable effect on host animal survival or behavior, but the effect on zooxanthellae was profound. Low temperature reduced the rate of photosynthesis, increased the rate of release of fixed carbon, reduced the number of viable cells and decreased the rate of cell division. Electron micrographs revealed that low temperature elicited the appearance of a thickened cell envelope and novel electron-dense inclusions, tentatively identified as crystallized lipoprotein. Immediately after low temperature incubation zooxanthellae move towards the apex of the host cells and are released to the coelenteron by exocytosis. The mechanisms by which low temperature may evoke exocytosis of zooxanthellae (*e.g.,* disassembly of host cell microtubules; increased concentration of cytosolic calcium ions) are discussed.

INTRODUCTION

Symbiotic dinoflagellates (=zooxanthellae) are found in tropical reef cnidarians (*e.g.,* sea anemones, corals) in very high concentration relative to the surrounding water column. Under normal conditions neither the algae nor the host outgrows the other, suggesting that the algal population density is somehow regulated (Muscatine *et al.,* 1985). Yet, when environmental conditions on coral reefs are naturally perturbed (lowered salinity, abnormally high or low water temperature, sub-aerial exposure, and UV irradiation), zooxanthellae are lost from corals (Goreau, 1964; Jaap, 1979, 1985; Egana and Disalvo, 1982; Verrill, 1902, and Mayer, 1914, in Porter *et al.,* 1982; Glynn, 1983, 1984; Lasker *et al.,* 1984; Harriott, 1985; Fisk and Done, 1985). Additional types of stress conditions, brought to bear on symbiotic cnidarians by investigators in the laboratory or in the field, also evoke loss of zooxanthellae. These conditions include constant light, elevated temperature, elevated salinity (Reimer, 1971; see also Steele, 1976, 1977), prolonged darkness (Yonge and Nicholls, 1931; Franzisket, 1970; Kevin and Hudson, 1979), shading (Rogers, 1979), and anoxia (Yonge and Nicholls, 1931; O. Hoegh-Guldberg, pers. comm.).

Given the fundamental importance of zooxanthellae to reef corals and other symbiotic cnidarians (Muscatine, 1980), it is surprising that very little is known of the mechanisms by which zooxanthellae population densities are controlled, or how the various environmental stresses evoke elimination of zooxanthellae.

Steen (1985) recently noted that exposure of the tropical symbiotic anemone *Aip-*

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tasia pulchella to low temperature (4°C) for one hour in darkness, followed by maintenance at 25°C in darkness, resulted in rapid and substantial loss of zooxanthellae over the next few days. The host was otherwise unaffected. In view of the rapidity of the response and the ability of the host to survive, we have used low temperature treatment as a bioassay for the study of mechanisms by which zooxanthellae are eliminated from their host.

This paper describes aspects of the kinetics and cell biological features of the loss of zooxanthellae from a sea anemone in darkness and after brief exposure to low temperature. The results show that although darkness evokes loss of zooxanthellae, low temperature accelerates loss four-fold, increases the permeability of zooxanthellae to photosynthate, and alters zooxanthellae ultrastructure. The mechanism by which low temperature evokes rapid exocytosis of zooxanthellae is discussed.

Materials and Methods

Organisms and maintenance

The tropical sea anemone *Aiptasia pulchella* (Clone B; Muller-Parker, 1984) was used for all experiments. Anemones were normally maintained in large finger bowls in 800 ml seawater in a Model 808 Precision Incubator at 25°C on a 12h:12h light: dark photoperiod (70 $\mu Ein \cdot m^{-2} \cdot s^{-1}$). Animals were fed freshly hatched *Artemia* nauplii twice weekly. After each feeding, seawater and uneaten *Artemia* were discarded and replaced with fresh seawater. When dark conditions were required, a similar incubator was used, without lights, and the temperature was adjusted to 25°C. Anemones were not fed during the experiments.

Counting numbers of zooxanthellae in sea anemones

Samples of five anemones of similar size were homogenized in a glass-Teflon Potter homogenizer (10 ml), and the homogenate centrifuged in an IEC-HNS table centrifuge at 700 × *g* for 3 min to pellet the algae. The animal homogenate was decanted and the pellet preserved in seawater containing 4% formaldehyde. Numbers of algae per anemone were determined from hemacytometer counts of samples of formalin-fixed cells. Algal pellets were saved and used later for determination of mitotic index (see below).

Viability of zooxanthellae expelled from anemones

Viability of zooxanthellae was assessed from levels of fixation of ^{14}C and from observations on dye exclusion.

Expelled clumps of zooxanthellae were collected with a Pasteur pipette from bowls in which anemones were maintained. When sufficient cells were collected, the clumps were gently homogenized in several ml seawater and 5 replicate samples of 1 ml each were placed in separate 12 ml conical graduated centrifuge tubes. Samples were taken for cell counts. Then, 0.1 ml of a stock solution of $NaH^{14}CO_3$ containing one μCi per ml was added to each tube. After mixing the contents of each tube, 25 μl of the stock solution was immediately taken for estimation of Added Activity. Tubes were incubated for 2 h at 300 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ at 25°C and mixed again after one h. After incubation, tubes were centrifuged at 1000 × *g* for 4 min to pellet the algae, and the labelled seawater supernatant was decanted and saved. The algae were resuspended in 0.2 ml distilled water, the suspension was transferred to glass scintillation vials, acidified with 0.1 ml 1 *N* HCl, and evaporated to dryness with low heat on a warming

plate. Samples of labelled supernatant were treated similarly. Then 10 ml scintillation cocktail was added to each vial. For Added Activity, samples were added to 10 ml of scintillation cocktail, and the vials were capped immediately. Vials were counted in a Beckman LSC Model 100 scintillation counter, and corrected for quench by the external standards ratio method. Results are expressed as μg C fixed $\cdot 10^6$ cells$^{-1} \cdot$ h^{-1}.

For the dye exclusion assay, expelled zooxanthellae were collected, and placed on a standard microscope slide. A drop of 0.25% (w/v) Evans Blue (Gaff and Okong'o-Ogola 1971) was added and the percentage of cells permeated by the dye after 5 min was established from observation of five replicate samples of 200 cells at 450×.

Mitotic index

Mitotic index was determined for zooxanthellae in the same samples used to measure zooxanthellae population density. The number of cells undergoing cytokinesis was determined for 10 samples of 100 cells, each viewed at 450× on a hemacytometer grid to facilitate counting.

Specific growth rates and loss rates of zooxanthellae

Specific growth rates of zooxanthellae *in situ* were calculated from

$$\mu = 1/N\ (N/t)$$

where N = the standing stock of zooxanthellae and (N/t) the increment of new cells added per day (Strickland and Parsons, 1965). By analogy, specific expulsion rates were calculated from

$$\mu_x = 1/N\ (N_x/t)$$

where N_x is the number of algae expelled, calculated from N at $t_0 - t + 10$ days, and N_x/t, the average increment of cells expelled per day (O. Hoegh-Guldberg, pers. comm.).

Morphology and ultrastructure

For transmission electron microscopy whole anemones and discharged pellets of algae were fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1 *M* phosphate buffer according to McDowell (1978). Tentacle crowns were removed and subdivided into small pieces, rinsed in buffer, and dehydrated in a series of ethanol solutions (30%, 50%, 70%, 90%, and three changes of absolute ethanol), 30 min at each step. Tissues were transferred to absolute ethanol:propylene oxide (1:1) for 1 h, then into propylene oxide:medcast epoxy (1:1) and finally into 100% medcast epoxy for infiltration overnight. Tissues were embedded in fresh 100% medcast and the material was hardened according to the manufacturer's instructions.

Thick sections of tentacles and pellets were cut with glass knives, stained with methylene blue and mounted on standard microscope slides. These were viewed and in some cases photographed with a Olympus BH-2 compound microscope using Panchromatic BW film (ASA 200). Some tentacles were deliberately cut in cross section to facilitate determination of the intracellular locus of individual algae with respect to the long axis of their host endodermal cell. This locus is expressed in terms of R_f, the ratio of the distance from (a) the base of the endoderm cell to the leading edge of the alga, to (b) the base of the endoderm cell to the center of the tentacle lumen. The latter criterion was used because the apex of each endoderm cell usually could not be discerned.

Thin sections of tentacles or algal pellets were cut with diamond or glass knives, collected on 200 mesh copper grids coated with parlodion and carbon film, and stained with uranyl acetate and lead citrate. Grids were examined with a Philips 300 Electron microscope operating at 60 kV.

For scanning electron microscopy, excised tentacles and discharged pellets were fixed in 4% formalin in seawater. Tentacles were slit longitudinally with a scalpel blade and all specimens were critical-point dried in a Polaraon Critical-Point Drier, mounted on stubs, sputter-coated with gold, and viewed with an ISI-DS-130 scanning electron microscope.

Results

The effect of darkness on maintenance of zooxanthellae population density

Since our bioassay was carried out in cold and darkness, it was necessary to quantify and thereby control for loss of algae from *A. pulchella* due to darkness alone. Groups of *A. pulchella* that were starved for one day were incubated for 4 h in darkness at 25°C. Thereafter they were maintained in darkness without feeding for up to 20 days. Five anemones were sampled at zero time (immediately after treatment) and at two-day intervals, and the number of zooxanthellae remaining in each anemone was determined. The results are shown in Figure 1. In these anemones, the numbers of algae decreased steadily, and over ten days the standing stock of algae decreased by 58%. ($\mu_x = 0.06\ d^{-1}$). Clumped pellets of zooxanthellae were found in the experimental containers in the vicinity of individual anemones during the experiment, indicating that the loss of substantial numbers of zooxanthellae was due to expulsion from the anemone. Digestion of some algae could have occurred as well, but this possibility was not investigated.

Effect of a brief exposure to low temperature on the maintenance of zooxanthellae population density

Anemones incubated at 14°C for 4 h in darkness and then maintained at 25°C in darkness lost zooxanthellae at a faster rate ($\mu_x = .08\ d^{-1}$) than controls, losing about 72% of their algae in 10 days (Fig. 1). But when anemones were incubated at 4°C in darkness and then 25°C in darkness, loss of zooxanthellae was even more rapid. After only 4 days the population of algae had decreased by 98% ($\mu_x = .24\ d^{-1}$). Further decrease in numbers was only slight over the next 20 days. The combination of darkness and low temperature incubation caused relatively rapid loss of roughly 98–99% of the algae associated with *A. pulchella*.

Low temperature incubation had no obvious visible effects on the host anemone behavior. They exhibited normal posture (column erect, tentacles extended) and feeding behavior, and continued to produce buds by pedal laceration for at least six months after low temperature treatment. However, in one case, several tentacle tips, 1–2 mm long and filled with zooxanthellae, were recovered from incubation dishes. Presumably these had sloughed or broken off during or after low temperature treatment.

Effect of light on loss of zooxanthellae after low temperature incubation

To determine if the loss of zooxanthellae after low temperature treatment in darkness was affected by light, a group of anemones was incubated at 4°C in darkness as usual, but then maintained in the light ($70\ \mu Ein \cdot m^{-2} \cdot s^{-1}$) for 10 days. Anemones were sampled at two-day intervals and numbers of zooxanthellae per anemone deter-

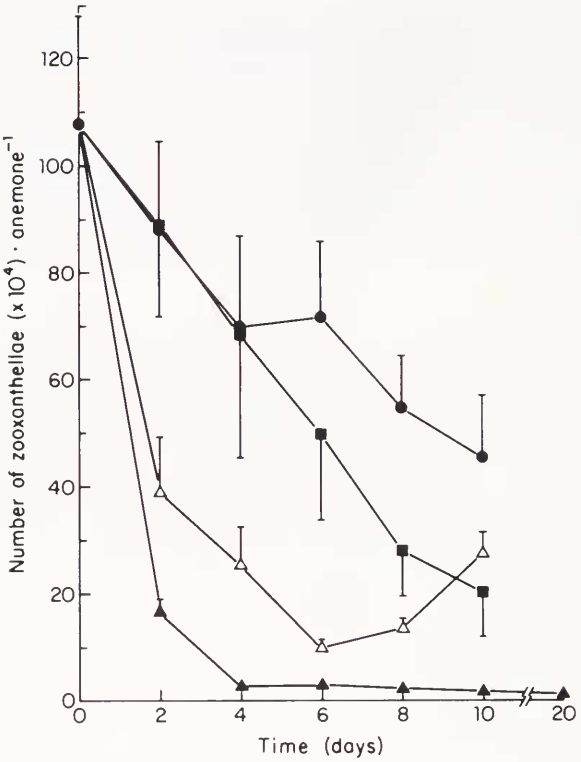


FIGURE 1. Number of zooxanthellae remaining in *Aiptasia pulchella vs.* time in dark at 25°C after an initial 4-h incubation at 25°C (●), 14°C (■), and 4°C (▲); and time in light at 25°C after an initial 4-h incubation in dark at 4°C (△). Vertical bars indicate standard error of the mean.

mined. Figure 1 shows that such individuals lost abundant zooxanthellae immediately and at approximately the same rate as those that were chilled and maintained in darkness, but the rate of loss decreased slightly after 2 days until, by day 6, about 91% of the original population had been expelled. Then, over the next 4 days, the population began to increase at a specific growth rate of about 0.44 d^{-1}.

Viability of cold-treated zooxanthellae

To determine if low temperature incubation affected the viability of zooxanthellae, we tested the ability of freshly isolated and expelled zooxanthellae to photosynthetically fix ^{14}C and to exclude dye. The results are given in Table I.

Zooxanthellae freshly isolated (FIZ) from control anemones (25°C, dark) fixed ^{14}C at normal rates (*cf.* Muller-Parker, 1984), and about 98–99% of these zooxanthellae were viable as judged from ability to exclude dye. FIZ from cold (4°C) treated anemones assayed immediately or after a 4-h recovery period also fixed ^{14}C but at much lower rates. Of these cells, 91.8–93.6% were judged viable. Extending the recovery period at 25°C to 24 h resulted in a modest increase in photosynthetic rates, but the rates were still below control levels and viability was reduced to 82.5%.

FIZ customarily release a fraction of their fixed ^{14}C to the external medium (Muscatine, 1980). Those from *A. pulchella* (Clone B) normally release less than 10% of

TABLE I

Photosynthetic rate ($\bar{x} \pm$ S.D.) and % viability (dye exclusion) of zooxanthellae freshly isolated (FIZ) and/or expelled (EZ) from control (25°C dark) and chilled (4°C dark) Aiptasia pulchella

Host treatment	Recovery period before assay	Cells assayed	Photosynthetic rate ($\mu gC \cdot 10^6$ cells$^{-1} \cdot h^{-1}$)	Viable cells (%)	Cell-specific photosynthetic rate ($\mu gC \cdot 10^6$ viable cells$^{-1} \cdot h^{-1}$)
25°C dark					
4 h	—	FIZ	9.63 ± 1.57	98.7	9.76
			4.15 ± 0.53	98.5	4.22
					$\bar{x}$ = 6.99 ± 3.92
4°C dark					
4 h	—	FIZ	0.51 ± 0.05	91.8	0.56
4 h	25°C 4 h, dark	FIZ	0.70 ± 0.09	93.6	0.74
4 h	25°C 24 h, dark	FIZ	2.89 ± 0.21	82.4	3.50
		EZ	1.53 ± 0.13	82.3	1.86
					$\bar{x}$ = 1.42 ± 1.02

the total ^{14}C fixed in a one-hour incubation (O. Hoegh-Guldberg, pers. comm.). Our controls released 28.8% under similar conditions (n = 5). In contrast, zooxanthellae from cold-treated anemones released an average of 58.8 ± 3.4% (n = 5) of the total ^{14}C fixed. Therefore, the cold treatment seemed to reduce rates of photosynthesis, increase the percentage of fixed ^{14}C released, and reduce numbers of viable cells.

Expelled zooxanthellae (EZ) were similar to FIZ with respect to ^{14}C fixation. Algae expelled up to four hours after cold treatment showed greatly reduced rates of photosynthesis and about 61% viability. Those expelled during a recovery period of 4–24 hours at 25°C showed some increase in rate of photosynthesis and level of cell viability, but the mean cell-specific rate of photosynthesis (1.42 ± 1.02 $\mu gC \cdot 10^6$ cells$^{-1} \cdot h^{-1}$) was still significantly lower than that of freshly isolated controls (6.99 ± 3.92; $P < 0.05$, Student's t-test). Release of fixed ^{14}C by EZ was not assayed.

Effect of low temperature on cell division

The mean MI for zooxanthellae from *A. pulchella* (Clone B) maintained normally is 0.76% (Wilkerson *et al.*, 1983), and ranges from 0.38 to 1.54% (Muller-Parker, 1984). Figure 2 shows that mitotic index of zooxanthellae from controls incubated at 25°C ranged from 1.65% at time zero to 4.6–5.1% after 2–4 days in darkness and then declined again to 2% after 10 days. In contrast, algal cells from anemones incubated at 14° or 4°C had lower mitotic indices, ranging between 1.25 and 0.25%. These results indicate that darkness gave rise to a transient increase in MI and that brief exposure to low temperature may inhibit such an increase. Zooxanthellae from anemones incubated in light at 25°C after low temperature incubation in darkness exhibit a higher mitotic index, suggesting that light may reverse the inhibitory effect of low temperature on cell division.

Morphological correlates of expulsion of zooxanthellae

Intracellular loci of zooxanthellae. Zooxanthellae are confined to the endoderm cells of *A. pulchella.* Most endoderm cells contain 1–3 algae, each within an animal cell vacuole (Glider *et al.*, 1980). To gain insight into morphological correlates of

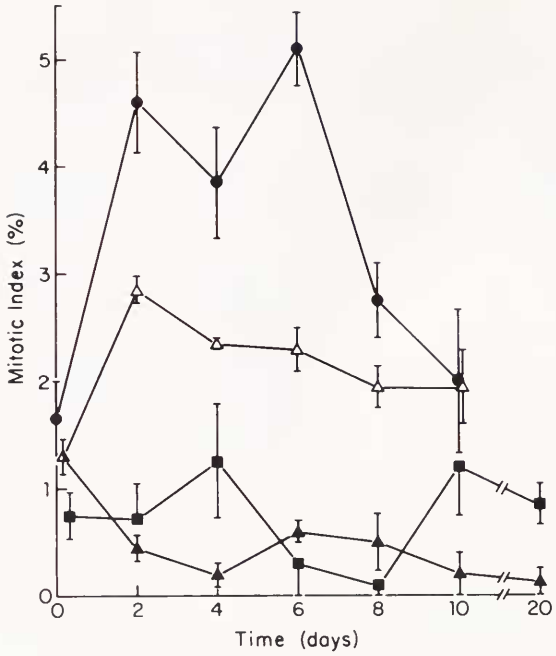


FIGURE 2. Mitotic index (%) of zooxanthellae in *Aiptasia pulchella vs.* time in dark (or light) at 25°C after initial treatment as in Figure 1. Symbols and vertical bars as in Figure 1.

expulsion, the loci of algae along the long axis of host endoderm cells, before and after exposure to darkness and low temperature, were determined from R_f measurements of 80–200 cells in thick sections of tentacles. Results are shown in Figure 3. Zooxanthellae in cells of controls maintained in darkness for 4 h at 25°C were evenly distributed along the long axis of host cells. During prolonged maintenance in darkness, the population shifted toward the apical end of the host cells. In cold-treated anemones, this shift was detectable even at time zero. Within 24 hours after the cold treatment, the pattern appears reversed, but it is likely that the zooxanthellae near the luminal face of the host cell had already been expelled. The apical shift is restored after two days and considerably exaggerated after four days. These data are consistent with the interpretation that a change in intracellular locus precedes and accompanies the discharge of zooxanthellae.

Ultrastructure of zooxanthellae in situ *and during expulsion.* Zooxanthellae *in situ* in anemones maintained in light at 25°C are shown in Figure 4a. The cell envelope consists of two to five sets of double membranes between the presumptive animal cell vacuolar and zooxanthellae plasma membranes. The average thickness of the envelope is about 60 nm. The chloroplast is lobed, with a prominent chloroplast membrane and lamellae in the three-thylakoid configuration. The pyrenoid (Fig. 4a, inset) is congruent with the chloroplast stroma but apparently not traversed by thylakoids. It is capped by a thick sheath, presumably consisting mainly of starch. The nucleus contains condensed chromosomes. Mitochondria are present and adjacent to the pyrenoid there is a large amorphous body and crystal-like electron translucent profiles.

Zooxanthellae *in situ* in the anemones incubated at 4°C and sacrificed at day 0

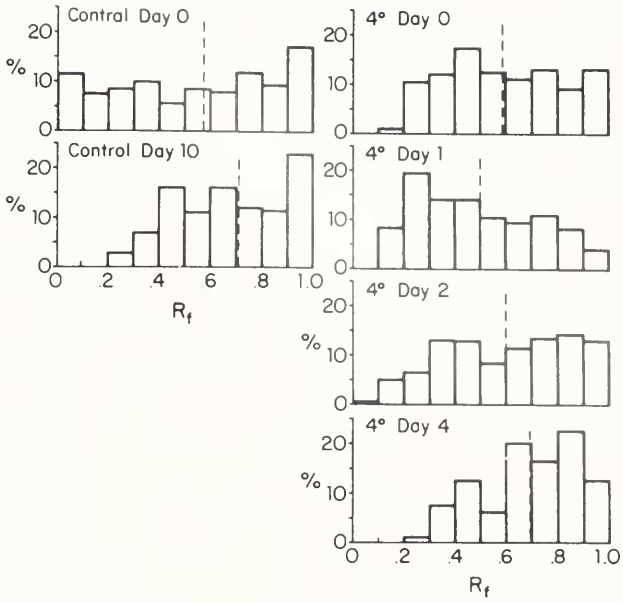
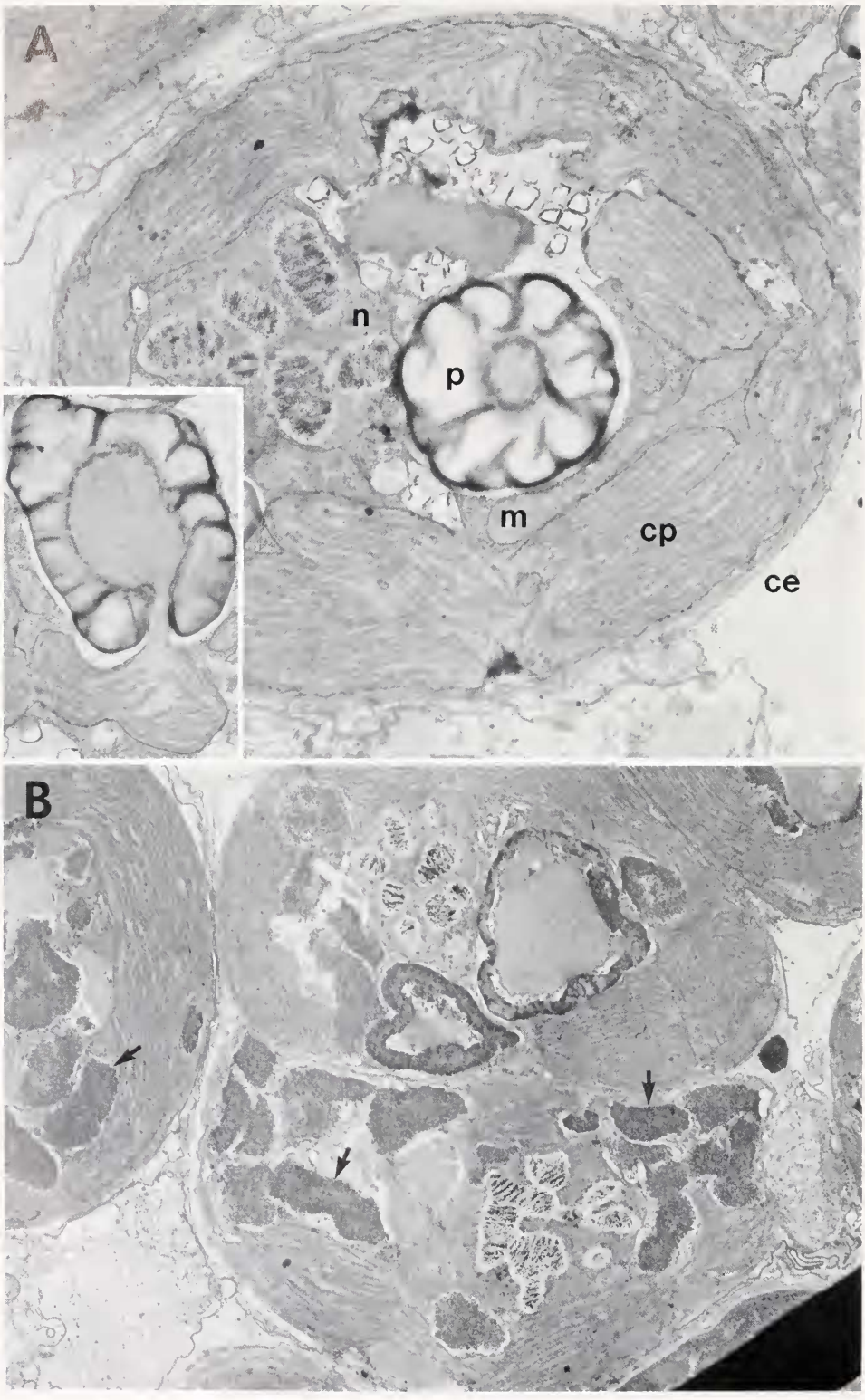


FIGURE 3. Number of zooxanthellae (%) *vs.* relative position along the long axis of host cells in control (25°C, dark 4 h) and chilled (4°C, dark 4 h) anemones. Dashed line indicates mean R_f.

(immediately after cold treatment) are shown in Figure 4b. In some cells the average thickness of the cell envelope has increased and there is loss of resolution of cell envelope membranes. The most striking change is manifested by the appearance of electron-dense inclusions and an increase in the density of the pyrenoid starch cap. After one day (Fig. 5a), cell envelope thickness has increased to about 120 nm. The dense inclusions are less granular and now exhibit a dense core. Dense core inclusions are also present in the host cell. Electron-dense inclusions are still evident in zooxanthellae in anemones sacrificed after two days (not shown).

Release of zooxanthellae from tentacle cells. The inner surface of tentacles is lined with flagellated endoderm cells, each containing zooxanthellae. In tentacles of control anemones, the zooxanthellae are situated proximally within the host cells and the endodermal epithelium is relatively smooth (not shown). As a result of chilling, the zooxanthellae move distally within the host cells, causing the distal end of the host cell to bulge into the tentacle lumen and to assume the spherical shape of the zooxanthellae they contain. Low power scanning electron micrographs of the tentacle endoderm immediately after chilling give the general appearance of a tentacle lined with extracellular zooxanthellae (Fig. 6a). However, at this time most of the zooxanthellae are still within the host cells. Figure 6b shows the flagella at the apex of the extended host cells and in one case a torn host cell shows that it persists as a thin enclosure for a zooxanthellae cell. About 8 h after chilling, zooxanthellae can be seen emerging from host cells, apparently by exocytosis (Fig. 6c). By 12 h the terminal stages of exocytosis and release of zooxanthellae can be observed (Fig. 6d). By 20 h most of the zooxanthellae have been discharged from the epithelium. Those few that remain are also in late stages of exocytosis (Fig. 7a).

Algae are discharged from the coelenteron as individual cells or as pellets of cells. The pellets are irregularly spherical, and also contain bacteria and assorted fibrils


A
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and sheets of material of unknown origin and composition (Fig. 7b). Transmission electron micrographs of pellets (Fig. 5b) show that some zooxanthellae are apparently intact and exhibit a thick cell envelope, while others are in various stages of disintegration. Each is surrounded by one or more thin membranes raised above the surface of the cell. Figure 7c shows that the algae are generally spherical but enclosed by a loose wrinkled sheath probably corresponding to the elevated membranes shown in Fig. 5b). In cells *in situ* this sheath is torn apart and shed as the two daughter cells undergo cytokinesis (Fig. 7d) and is therefore interpreted as a mother cell envelope.

Discussion

The effect of darkness on maintenance of zooxanthellae population density

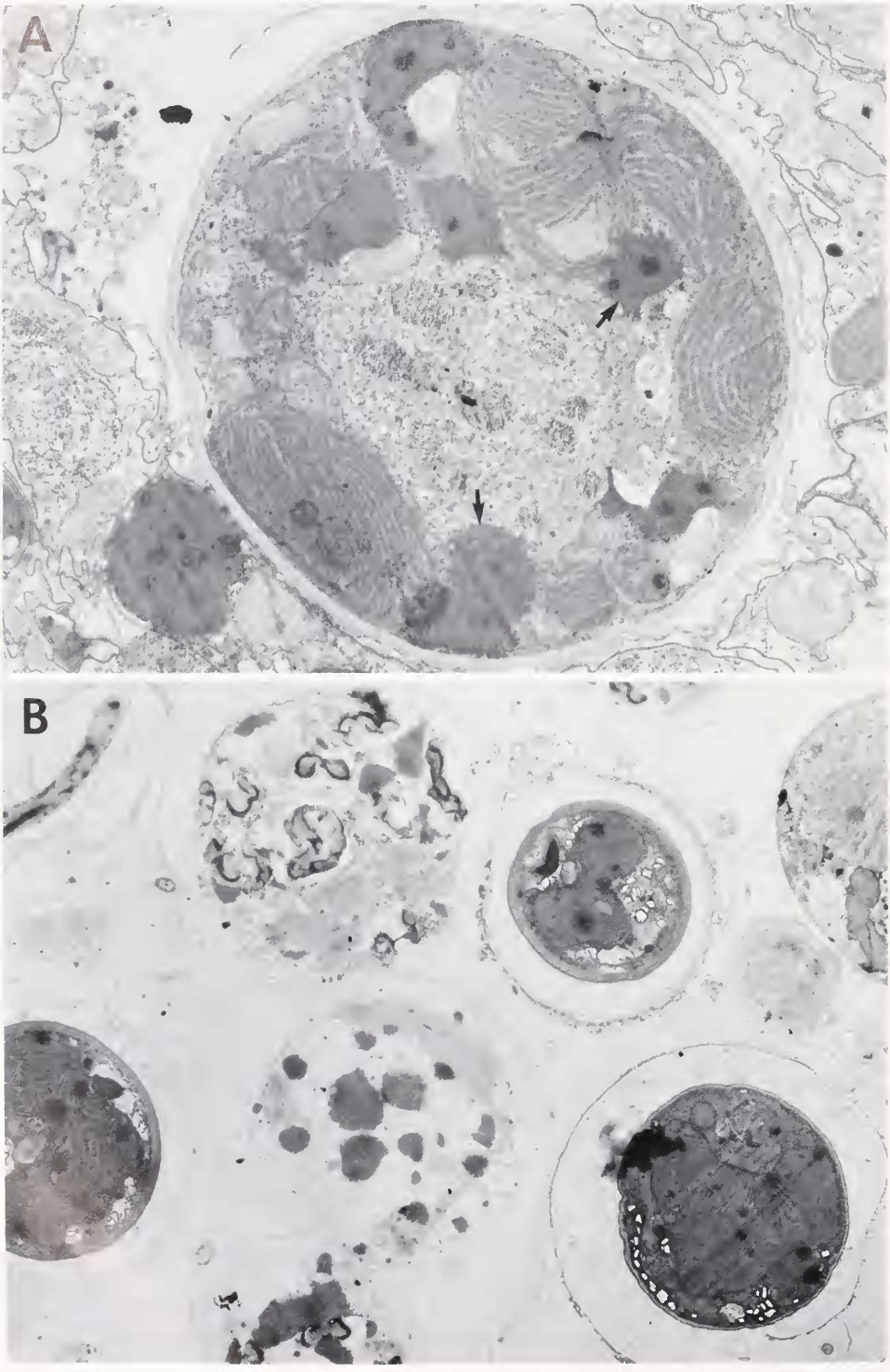
The results of this investigation show that when the symbiotic anemone *Aiptasia pulchella* (Clone B) is maintained in darkness, without feeding, it progressively loses zooxanthellae. These results confirm the observations of Muller-Parker (1984), that darkness causes loss of zooxanthellae from *A. pulchella* (Clone B), and extend them by showing that at least 50% of the zooxanthellae are lost in 10 days.

Previous studies have demonstrated that symbiotic anemones and corals lose zooxanthellae in prolonged darkness (Yonge and Nicholls, 1931; Buchsbaum, 1968; Franzisket, 1970; Kevin and Hudson, 1979; Rogers, 1979; Muller-Parker, 1984), but neither the time course nor the mechanism of loss has been rigorously investigated. More recently, Glider (1983) demonstrated that symbiotic *Aiptasia pallida* maintained on a 12 h light:12 h dark photoperiod continuously extrude pellets of zooxanthellae but in significantly greater numbers during the dark period.

In the case of *A. pulchella,* loss of zooxanthellae in darkness could occur by expulsion or digestion. The appearance of numerous clumps of zooxanthellae in the vicinity of individual anemones shows that loss is due, at least in part, to expulsion. Data from Figure 3 show that algae are initially distributed more or less evenly along the long axis of the host cell. In darkness, they move toward the distal end of the host cell and are then released from host cells by exocytosis (Fig. 6c, d). In the coelenteron they form large pellets which are then egested. Egestion of regularly formed pellets of zooxanthellae by *A. pallida* and by Hawaiian zoanthids has been described by Reimer (1971). The mechanism which evokes intracellular migration and exocytosis of zooxanthellae in darkness is unknown but may be correlated with diminished algal photosynthesis. In this connection, we note that the maintenance of symbiotic *Chlorella* sp. in the cells of green hydra may depend on the sustained translocation of photosynthetically fixed carbon from the algae to the host cells (Hohman *et al.*, 1982).

There is evidence for presumed digestion of zooxanthellae in some symbiotic cnidarians (Fitt and Trench, 1983) but the possibility was not investigated in this study. The remainder of this discussion will address loss of zooxanthellae by expulsion only.

FIGURE 4. (a) Transmission electron micrograph of a zooxanthella in *Aiptasia pulchella* maintained at 25°C on a 12L:12D photoperiod. ce, multilayered cell envelope; cp, chloroplast; m, mitochondrion n, nucleus with condensed chromosomes; p, pyrenoid, 20,383×. Inset, chloroplast-pyrenoid complex, 17,976×. (b) Zooxanthella from an anemone maintained 4 h at 4°C in dark. Electron-dense inclusions at arrows. 11,194×.


A
B

The effect of low temperature on maintenance of zooxanthellae population density

Brief exposure to low temperature increased the rate of loss of zooxanthellae slightly at 14°C but four-fold at 4°C (Fig. 1). We speculate that low temperature could potentiate exocytosis by increasing the rate of microtubule depolymerization (Melkonian *et al.*, 1980; Dustin, 1984). For example, in green hydra, endoderm cells form endocytic vacuoles upon uptake of symbiotic *Chlorella* sp. These vacuoles migrate away from the site of uptake (Muscatine *et al.*, 1975). Migration is thought to be mediated by microtubules since the movement of vacuoles containing algae is inhibited by colchicine (Cook, unpub., in Muscatine *et al.*, 1975) or vinblastine (McAuley and Smith, 1982). Treatment of green hydra with vinblastine, which promotes microtubule disassembly, causes the algae, which are normally situated at the base of the host cell, to gradually shift back toward the apex of the cell (L. Muscatine, unpub.). Low temperature could also stimulate exocytosis by causing an increase in cytosolic calcium ions (Campbell, 1983). Increased cytosolic calcium has been implicated in a wide range of systems involving stimulus-secretion coupling (See, for example, Douglas, 1973; Dahl and Henquin, 1978; Spearman and Butcher, 1982).

Effect of light on loss of zooxanthellae after low temperature incubation

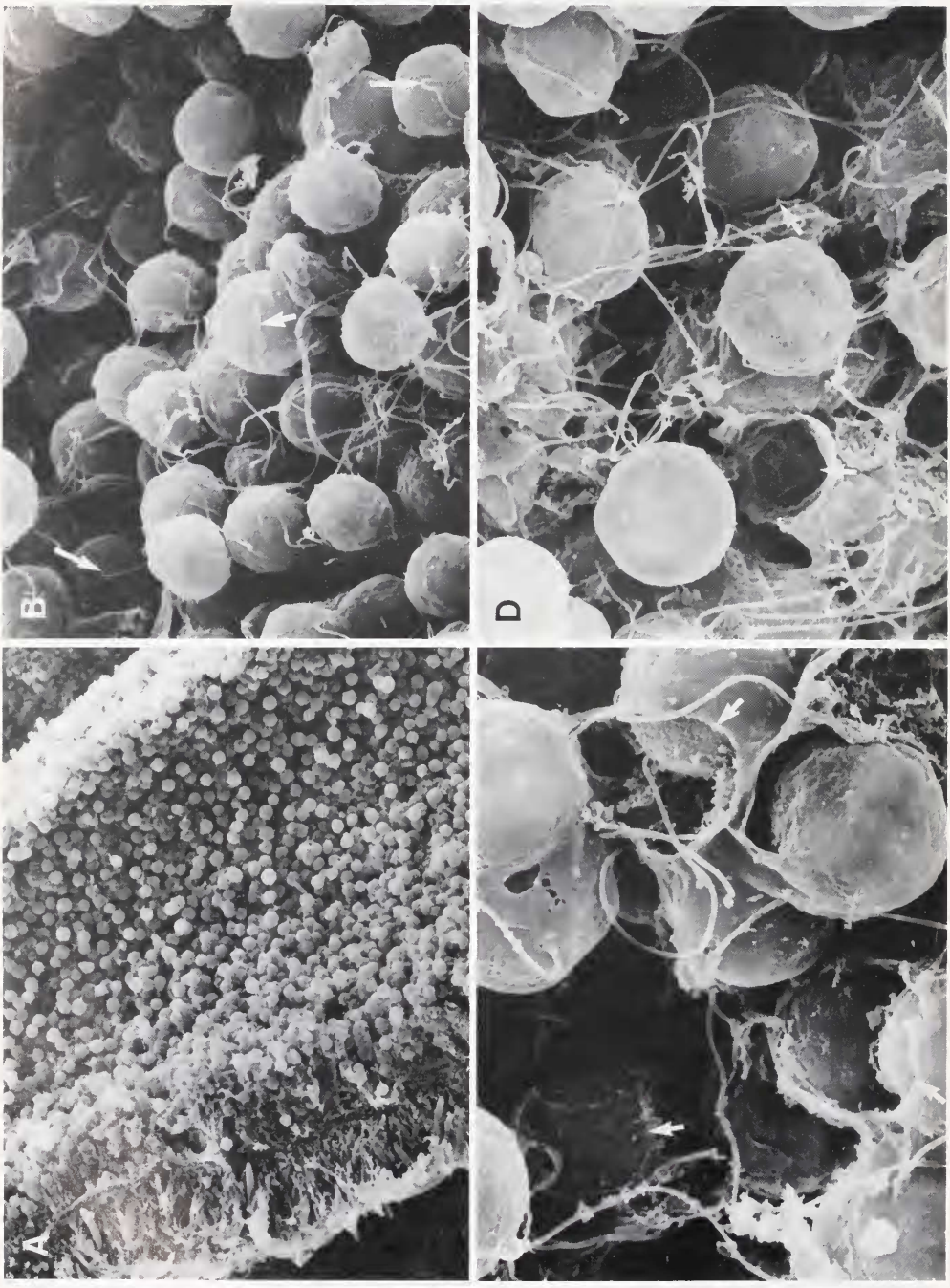
Maintenance of *A. pulchella* in light after low temperature incubation in darkness did not significantly affect loss of zooxanthellae for up to six days. This suggests that the effect of low temperature incubation is not quickly or easily reversed by the restoration of photosynthesis or translocation, or the putative reassembly of microtubules. After six days, the standing stock of algae begins to increase, suggesting that the effect of low temperature is no longer manifested.

Viability of cold-treated zooxanthellae

Zooxanthellae expelled by *A. pallida* are photosynthetically active (Glider, 1983), as are zooxanthellae freshly isolated from *A. pulchella* after incubation for 4 h in darkness at 25°C (Table I). However, incubation of *A. pulchella* at 4°C caused a decrease in photosynthetic rate and impaired the ability of freshly isolated or expelled cells to exclude dye. This effect of low temperature was only partially reversible at 24 h, again suggesting that metabolism was substantially altered. This conjecture is supported by data showing that release of fixed ^{14}C by chilled cells was higher than release by controls. Table I also shows that, since the percentage of viable cells in samples of expelled zooxanthellae was less than the percentage in freshly isolated zooxanthellae, the cells impaired by chilling may be preferentially expelled.

Interestingly, the viability of zooxanthellae freshly isolated from controls was 98.6%. These cells were separated from the host by homogenization and centrifugation. However, if cells are isolated by excising a tentacle and gently pressing it with a glass rod, then 100% of the cells are able to exclude the Evans Blue dye. This implies that either a small proportion of the zooxanthellae are damaged by the homogenization method or that non-viable cells are derived from other parts of the anemone.

FIGURE 5. (a) Transmission electron micrograph of a zooxanthella from an anemone incubated 4 h in dark at 4°C, then 24 h in dark at 25°C. Electron-dense inclusions at arrows. 18,846×. (b) Transmission electron micrograph of a pellet of zooxanthellae discharged after 5 days from anemones treated as in 5a. 7780×.


A
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D

Effect of low temperature on cell division

The MI of zooxanthellae increased from the normal range of 0.38% to 1.54% to 4.6% to 5.1% after several days in darkness. The reason for this increase is not yet known, but a similar result was obtained by Glider (1983). He found that MI was greater in darkness in zooxanthellae both *in situ* in *A. pallida* and in expelled pellets. Given that algae are expelled in darkness, the effect of increased division rate in darkness is to offset the rate of expulsion. Since low temperature decreases MI (Fig. 2) it is possible that accelerated loss arises merely from a reduction in the rate of replacement. Calculations addressing this possibility (Appendix 1) suggest that it is highly unlikely that accelerated loss arises solely from inhibition of division (replacement) rate.

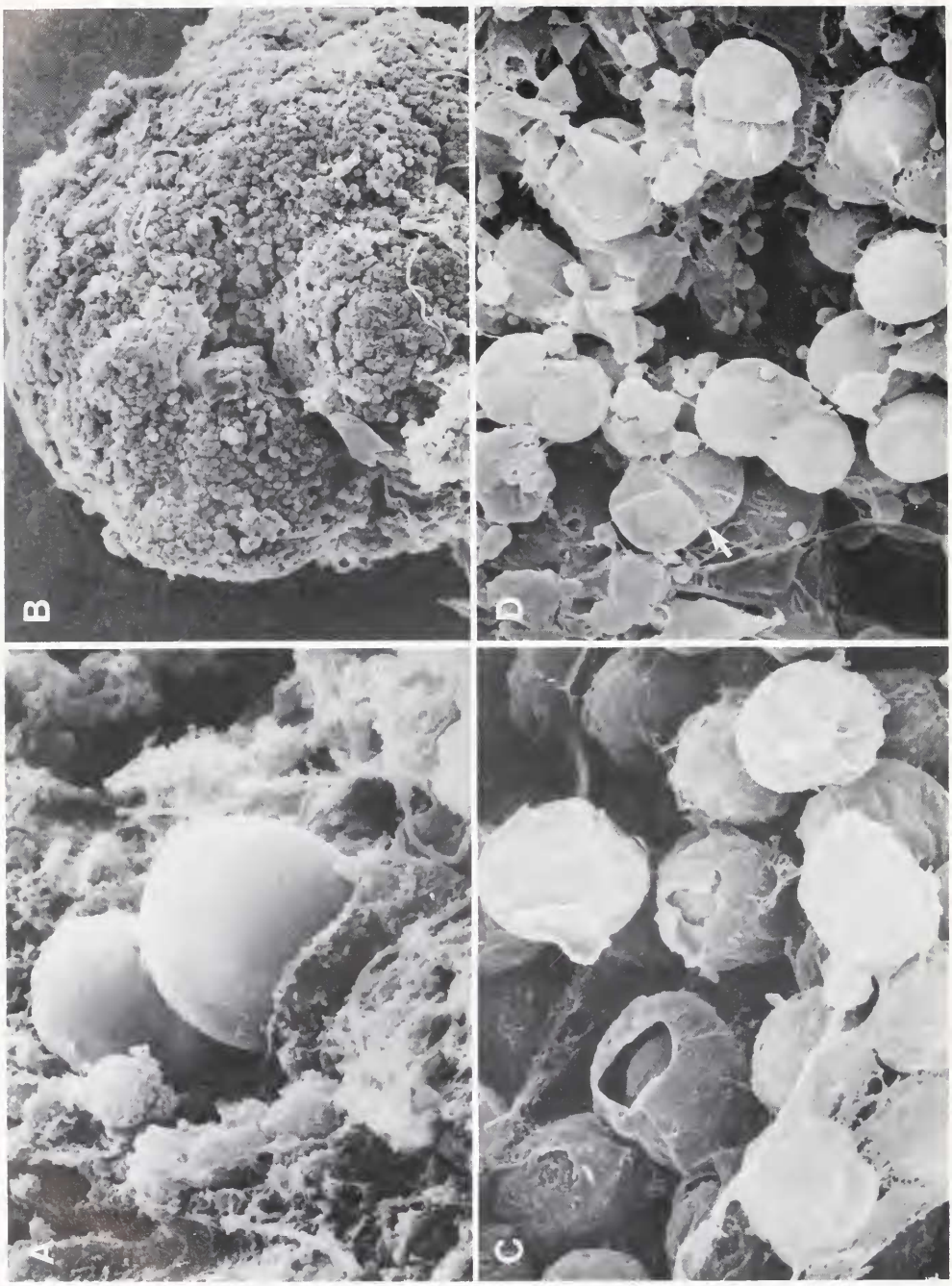
Morphological correlates of expulsion of zooxanthellae

Transmission electron micrographs of chilled zooxanthellae *in situ* show a thickened cell envelope. The significance of the thickened envelope is not yet understood but it does resemble the "pellicle" which develops when zooxanthellae are grown in culture (Schoenberg and Trench, 1980; Glider, 1983). The nature of the electron-dense entities in zooxanthellae cytoplasm which do not appear in controls is unknown. Puppione *et al.* (1982) suggest that triglyceride-rich lipoprotein enriched in saturated fats, upon exposure to temperatures sufficiently low to induce phase transition, forms crystallized particles enclosed within a phospholipid monolayer. If zooxanthellae contain such materials in membranes, and if they crystallize at low temperature, membrane function might be perturbed. Such a perturbation might explain the observed increased release of photosynthate by chilled cells. In this connection, Harnischfeger and Jarry (1982) report that cooling *Chlorella emersonii* to around −5°C causes a reversible alteration in thylakoid fluidity which in turn increases the permeability of thylakoids to cations.

Glider (1983) observed transmission electron micrographs of host cells during normal expulsion of zooxanthellae. Algae at the distal end of host cells were covered with a thin layer of cytoplasm connected to the cell by a narrow tissue bridge. This profile was interpreted as imminent "budding" rather than classical exocytosis. The animal tissue covering was thought to be digested in the coelenteron since it was not present in zooxanthellae pellets. We suggest that Glider (1983) observed the apical "bulging" of host cells caused by the extreme distal migration of zooxanthellae. While it is possible that some zooxanthellae are released by "budding," our scanning electron micrographs (Figs. 6b–d) offer direct evidence to support the interpretation that zooxanthellae are discharged by exocytosis. Further, the loose, wrinkled sheath which we observed in pellet zooxanthellae (Fig. 7c) more likely corresponds to a mother cell envelope, since it seems to be intimately associated with the cells during cytokinesis.

Studies now in progress show that brief exposure to low temperature evokes exocytosis of zooxanthellae in *A. pallida* from Bermuda and *Aiptasia* sp. from Java, but not in several scleractinian corals from Bermuda. The specificity of this phenomenon is now under investigation.

FIGURE 6. (a) Inner surface of *Aiptasia pulchella* tentacle immediately after chilling, showing endoderm and abundance of zooxanthellae. 234×. (b) As above, showing that zooxanthellae are still within host cells. Host cell flagella and torn host cell revealing zooxanthella surface at arrows. 2400×. (c) As above, 8 h after chilling, showing zooxanthellae within and emerging from host cells (at arrows). 4750×. (d) As above, 12 h after chilling, showing zooxanthella freed from exocytotic cup (arrows). 3250×.


B
D
A
C

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APPENDIX 1

Let N_t = standing stock of algae per anemone at time t

$$\frac{dN_t}{dt} = \text{number of algae produced per unit time} - \text{number of algae expelled per unit time}$$

Let m = number of algae produced per alga per unit time, and

let x = probability of an algae being expelled per unit time.

Then,

$$\frac{dN_t}{dt} = N_t(m - x)$$

and since

$$\frac{dN_t}{dt} \cdot \frac{1}{N_t} = \mu, \qquad \text{then}$$

$$\mu = m - x$$

Our task is to compute

$$\mu_{25} = m_{25} - x_{25}$$

and $\mu_4 = m_4 - x_4$, and to compare x_{25} and x_4.

From Figure 1 we know N_t, so we can calculate μ at 25° and 4°C from

$$N_t = N_0 e^{\mu t}$$

This yields

$$\mu_{25} = -.08, \text{ and } \mu_4 = -.92$$

Then,

$$m = 1/t_d \ln(1 + f),$$

where t_d = duration of cytokinesis and f = the average daily mitotic index. Assuming that t_d is the same at 25° and 4°C, and taking the value of t_d = 0.46 days (Wilkerson *et al.*, 1983), then from Figure 2

$$f_{25} = 3.33\% \text{ and } f_4 = 0.54\%,$$

and

$$m_{25} = 0.71 \text{ and } m_4 = 0.12$$

FIGURE 7. (a) Endodermal surface of tentacle of *Aiptasia pulchella*, 20 h after chilling, showing a pair of zooxanthellae in late stage of exocytosis from host cell, and paucity of zooxanthellae elsewhere. 5910×. (b) Pellet of zooxanthellae discharged by chilled anemone. 241×. (c) Zooxanthellae in discharged pellet, each with a wrinkled envelope and connected by mucus strands. 3590×. (d) Zooxanthellae in tentacle lumen of anemone treated 4 h, 4°C in dark, showing various aspects of zooxanthellae mother cell envelope during cytokinesis. 2430×.

Therefore,

$$x_{25} = m_{25} - \mu_{25}$$
$$= .07 - (-.08)$$
$$= .15$$

and

$$x_4 = .01 - (-.92)$$
$$= .93$$

Therefore the probability of a zooxanthella being expelled after chilling is six times higher than that at 25°C.

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